Filovirus-like particles as vaccines and discovery tools

Kelly L Warfield, Dana L Swenson, Gretchen L Demmin and Sina Bavari[†]

Ebola and Marburg viruses are members of the family *Filoviridae*, which cause severe hemorrhagic fevers in humans. Filovirus outbreaks have been sporadic, with mortality rates currently ranging from 30 to 90%. Unfortunately, there is no efficacious human therapy or vaccine available to treat disease caused by either Ebola or Marburg virus infection. Expression of the filovirus matrix protein, VP40, is sufficient to drive spontaneous production and release of virus-like particles (VLPs) that resemble the distinctively filamentous infectious virions. The addition of other filovirus proteins, including virion proteins (VP)24, 30 and 35 and glycoprotein, increases the efficiency of VLP production and results in particles containing multiple filovirus antigens. Vaccination with Ebola or Marburg VLPs containing glycoprotein and VP40 completely protects rodents from lethal challenge with the homologous virus. These candidate vaccines are currently being tested for immunogenicity and efficacy in nonhuman primates. Furthermore, the Ebola and Marburg VLPs are being used as a surrogate model to further understand the filovirus life cycle, with the goal of developing rationally designed vaccines and therapeutics. Thus, in addition to their use as a vaccine, VLPs are currently being used as tools to learn lessons about filovirus pathogenesis, immunology, replication and assembly requirements.

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[†]*Author for correspondence*
United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702–5011, USA
Tel.: +1 301 619 4246
Fax: +1 301 619 2348
sina.bavari@det.amedd.army.mil

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Ebola (EBOV) and Marburg (MARV) viruses are the only members of the family *Filoviridae*, and were named according to their filamentous shape. EBOV and MARV cause acute and rapidly progressive hemorrhagic fever with mortality rates in humans of up to 90% [1,2]. The reservoir for filoviruses is unknown, although the consumption of monkey meat is often associated with onset of disease. Animals ranging from insects to mammals have been analyzed in the hopes of identifying a carrier, but to no avail [3]. After exposure, the onset of clinical symptoms can be as short as 2 days and as long as 21, although most infected humans and nonhuman primates die within 7–10 days of exposure [4]. In addition to hemorrhage and bleeding, other symptoms of filovirus infection include fever, headache, generalized myalgia, prostration, conjunctivitis, rash, lymphadenopathy, pharyngitis and edema [5].

Filoviruses are considered serious public health threats, and are classified as biosafety level (BSL)-4 agents and Category A biothreat agents by the US Centers for Disease Control and Prevention [6,101]. Features that characterize filoviruses as a significant global health risk include high morbidity and mortality rates, potential for person-to-person transmission, relative stability in the environment, and feasibility of large-scale production. Additionally, the filoviruses have a low infectious dose, an extremely rapid rate of replication and can be easily transmitted, including via aerosols [4,7,8]. Bolstering the fear of their use as bioterrorism agents, several hemorrhagic fever viruses have a history of state-sponsored weaponization, including EBOV and MARV [4,7,8]. These combined factors make members of the family *Filoviridae* extremely dangerous from both a public health and a bioweapon perspective. There

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14. ABSTRACT

Ebola and Marburg viruses are members of the family Filoviridae, which cause severe hemorrhagic fevers in humans. Filovirus outbreaks have been sporadic, with mortality rates currently ranging from 30 to 90%. Unfortunately, there is no efficacious human therapy or vaccine available to treat disease caused by either Ebola or Marburg virus infection. Expression of the filovirus matrix protein, VP40, is sufficient to drive spontaneous production and release of virus-like particles (VLPs) that resemble the distinctively filamentous infectious virions. The addition of other filovirus proteins, including virion proteins (VP)24, 30 and 35 and glycoprotein, increases the efficiency of VLP production and results in particles containing multiple filovirus antigens. Vaccination with Ebola or Marburg VLPs containing glycoprotein and VP40 completely protects rodents from lethal challenge with the homologous virus. These candidate vaccines are currently being tested for immunogenicity and efficacy in nonhuman primates. Furthermore, the Ebola and Marburg VLPs are being used as a surrogate model to further understand the filovirus life cycle, with the goal of developing rationally designed vaccines and therapeutics. Thus, in addition to their use as a vaccine, VLPs are currently being used as tools to learn lessons about filovirus pathogenesis, immunology, replication and assembly requirements.

15. SUBJECT TERMS

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are no vaccines available for preventing or treating EBOV or MARV infections at this time; thus, they have the priority for development of effective vaccines and therapeutics [9].

Biology of filovirus infections

Filoviruses are encoded by a 19 kb linear negative-sense RNA genome (FIGURE 1A). EBOV and MARV have simple genomes that encode seven proteins, with the EBOV genome also encoding for a single additional nonstructural protein [10–12]. Within the nucleocapsid of each virion, a single copy of the viral genome is associated with the nucleoprotein (NP), virion protein (VP)30 and VP35 (FIGURE 1B). The nucleocapsid is enclosed within a host-derived lipid bilayer containing the viral glycoprotein (GP) and matrix proteins VP24 and VP40 [13–17]. Oligomers of VP40 associate with microtubules, suggesting that VP40 traffics along microtubules to lipid rafts where assembly occurs [18–21]. VP40 alone is sufficient to drive virus-like particle (VLP) budding from the rafts, although addition of NP, VP24 and GP facilitates more efficient virion budding [22]. GP is cleaved by a furin-like enzyme into two fragments (GP_1 and GP_2) to produce the mature $GP_{1,2}$ [23–25]. The EBOV nonstructural protein, secreted (s)GP, is an N-terminal fragment lacking the transmembrane region required for membrane insertion [23,26]. Large amounts of sGP are found in the blood of infected animals and humans, but the exact role of sGP in EBOV pathogenesis is unknown at this time; both sGP and GP are thought to induce cytopathic changes, as well as host immune suppression [27].

The filovirus genome is not infectious because the host cell machinery is unable to support transcription or replication of the negative-sense RNA genome; therefore, filoviruses must carry all the necessary replication machinery within the virion [28,29]. The RNA-dependent RNA polymerase protein (L), NP, VP30, and VP35 form a ribonucleoprotein complex with the genomic RNA [30]. The filovirus replication strategy is thought to be similar to that of other negative-sensed RNA viruses, such as that of rhabdovirses and paramyxoviruses [31]. Antigen-presenting cells such as monocytes, macrophages and dendritic cells (DCs), appear to be the primary targets of filovirus infection, although filoviruses are known to infect almost any cell type tested, except B-, T-, and natural killer (NK) cells [32–42]. Several cellular coreceptors have been proposed to be involved in binding and entry of EBOV and MARV, including several C-type lectins such as dendritic cell–specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin (DC-SIGN) [35,43–47]. However, the exact nature of and requirement for these cellular receptors is unknown, and it is possible that the heavily glycosylated GP alone can bind and mediate entry via multiple cell-surface lectins [48–51].

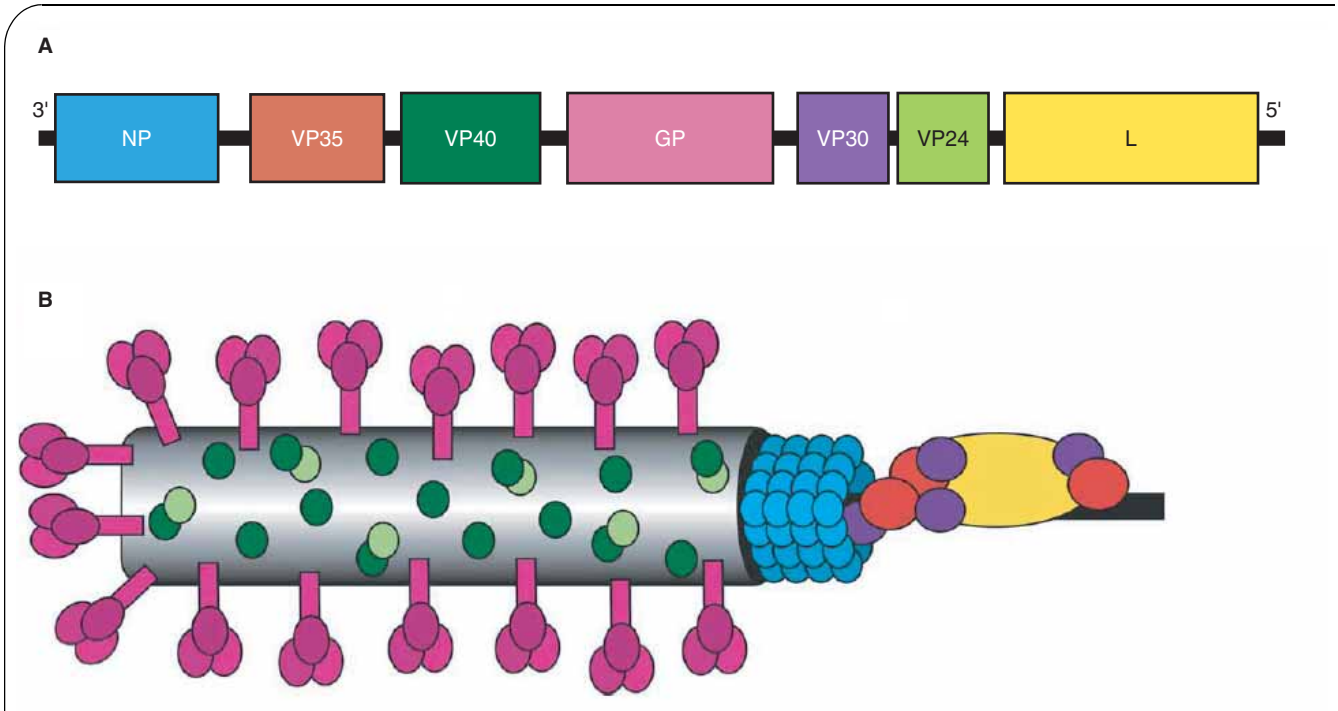

Figure 1. The genome and virus structure of the *Filoviridae*. (A) Linear representation of the filovirus genome. The Ebola and Marburg virus genomes are encoded by a single, negative-sense, linear strand of RNA, which is approximately 19 kb long. Both genomes encode for seven structural proteins (in the order NP, VP35, VP40, GP, VP30, VP24) and the L RNA-dependent RNA polymerase. Ebola virus also encodes for an additional nonstructural protein, secreted GP, which is not shown. (B) Schematic of the filovirus virion. The trimeric GP and matrix proteins VP24 and VP40, are associated with a host-derived membrane. The NP, VP30, VP35, and L associate with the viral RNA to form the ribonucleoprotein complex.
GP: Glycoprotein; L: Large RNA-dependent RNA polymerase; NP: Nucleoprotein; VP: Virion protein.

Vaccine approaches for filoviruses

To date, no vaccine has been licensed for protection against any of the EBOV or MARV strains. Classic methods for vaccine development have been tried, including producing and testing attenuated and inactivated viral preparations. A plaque-picked isolate of MARV that causes viremia, but not clinical signs or lethal disease, can protect guinea pigs against an otherwise lethal MARV challenge [52]. However, an attenuated virus vaccine is undesirable for filoviruses due to the danger of reversion to wild-type virulence. Similarly, a γ-irradiated whole-virion preparation has little potential for use in humans, as a single virion is sufficient to cause lethal disease. Additionally, vaccination with inactivated EBOV or MARV yields virus-specific antibodies, but fails to completely protect nonhuman primates from lethal challenge [53–59].

Several virus-vectored vaccine approaches have been tested for protection against filoviruses. Live vaccinia virus recombinant vaccines expressing EBOV sGP, GP, NP, VP35 or 40 were produced. Only the vaccine expressing EBOV GP protected guinea pigs, although vaccination with the vaccinia virus expressing GP did not protect cynomolgus macaques [56,60]. Recently, a live vesicular-stomatitis virus vaccine expressing GP was demonstrated to protect mice from lethal EBOV infection [61]. Of the most well-studied vectored vaccine approaches for filoviruses are the Venezuelan equine encephalitis (VEE) replicon particle (VRP), in which the antigen of interest is individually inserted in place of VEE structural genes [59,60,62–65]. VRP vaccines encoding GP have been the most successful of the six structural proteins tested, which also include NP, VP24, 30, 35 and 40 [10,52,62,63,65]. The VRP vaccine encoding GP is sufficient to completely protect nonhuman primates against MARV, although these animals were only partially protected against EBOV when vaccinated with 10^7 plaque-forming units (pfu) [56,65]. Sequential administration of a DNA vaccine and more than 10^{10} pfu of a defective adenovirus-vectored vaccine expressing both GP and NP, or the adenovirus vaccine alone, protected nonhuman primates against an EBOV challenge [66–68]. Several virus-vectored filovirus vaccines are candidates for further testing and evaluation, especially for determining mechanisms, correlates and predictors of immunity. However, questions regarding these strategies still remain, including concerns about residual replication-competent virus within vaccine preparations, acceptable vaccine doses, vaccine safety, and the impact of prior immunity to the vaccine vector.

Several nonvectored approaches have also been tested as filovirus vaccines. For EBOV and MARV, a prime-boost strategy with the DNA vaccine encoding GP and baculovirus-produced GP, completely protected animals from a lethal viral challenge [52,69]. However, vaccinating guinea pigs or nonhuman primates with either the baculovirus-produced GP, or DNA vaccine alone is not completely efficacious [52,69,70]. Although most of the work on developing filovirus vaccines has utilized virus vectors, it is clear from these studies that subunit vaccines have the potential to safely and specifically provide protection against lethal filovirus infection.

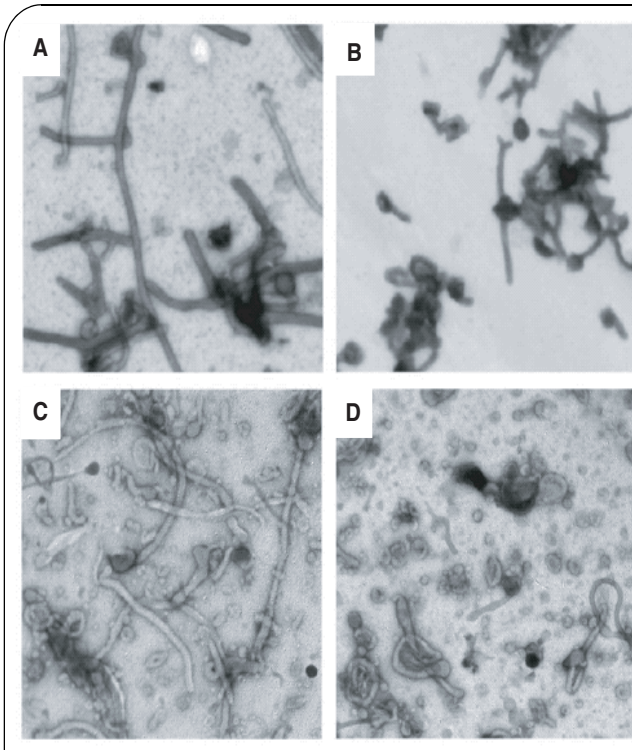

Figure 2. Virus-like particles are morphologically similar to authentic filovirus virions. Electron micrographs of (A) Ebola or (B) Marburg virus or virus-like particles from (C) Ebola or (D) Marburgat 12,000 ×. Particles were obtained by ultracentrifugation of the supernatants of VeroE6 cells infected with Ebola or Marburg virus (A-B) or 293T-cells transfected with both Ebola or Marburg glycoprotein and virion protein 40 (C-D). The samples were negatively stained with uranyl acetate to reveal the ultrastructure.

Generation of Ebola & Marburg virus-like particles

For many viruses, expression and production of the correct structural proteins is sufficient for forming VLPs. This is true for both nonenveloped viruses, such as parvovirus, papillomavirus, rotavirus and Norwalk virus, as well as enveloped viruses including influenza and HIV [71,72]. The generation of VLPs for both EBOV and MARV have been described in recent studies [15–17,20,21]. Morphologically, the filovirus-like particles are difficult to differentiate from authentic EBOV or MARV by either electron microscopy (FIGURE 2) [15–17,20,21] or atomic force microscopy [42,73].

Expression of the matrix protein VP40 alone is sufficient to drive formation of filamentous, enveloped VLPs which are released from cells [15,74]. VP40 may be required for the formation of VLPs, as other filovirus structural proteins alone, including GP, NP, VP24, 30 or 35, do not induce the formation of distinctly filamentous VLPs [UNPUBLISHED DATA]. However, optimal VLP formation and release requires the presence of additional viral proteins, especially VP24, GP and NP [17,20,21,74]. Release of both authentic filovirus virions and VLPs is also dependent on the integrity of lipid raft microdomains [20]. The GP traffics to cellular lipid rafts by acylation of

dual cysteine residues at the C-terminus of the transmembrane domain of GP where budding of VP40 from the cell surface through lipid rafts adorn the viral particle with GP spikes [20,75].

Structural requirements for filovirus assembly are only beginning to be understood. EBOV GP can be incorporated onto murine leukemia virus particles [76], and the authors of this review have recently demonstrated that hybrid VLPs can be constructed which contain EBOV GP and MARV VP40, or MARV GP and EBOV VP40 [60,77]. These hybrid VLPs display morphology similar to that of wild-type VLPs containing the homologous proteins, and also to the authentic filoviruses [77]. Furthermore, GPs from multiple EBOV and MARV subtypes can be incorporated onto Ebola VLPs using EBOV VP40 [78]. Taken together, these data suggest that the incorporation of viral proteins from different virus species and strains into VLPs is somewhat promiscuous.

Virus-like particles as filovirus vaccines

VLPs have been generated for many viruses, including rotaviruses, HIV, parvoviruses and human papillomavirus, by expressing select viral proteins in insect or mammalian cells [72]. Since VLPs are morphologically similar to the replication competent virus from which they are derived, the viral antigens are presented in a similar manner to the immune system and are highly immunogenic [71,79,80]. VLPs potently stimulate functional maturation and activation of DC, key cells responsible for subsequent activation of both humoral and cellular immune responses [81–83].

Ebola virus-like particles

Enveloped Ebola (e)VLPs have been generated in a mammalian expression system, and the purified VLPs containing GP and VP40 from Ebola used as vaccines [20,21]. Following eVLP vaccination, BALB/c and C57Bl/6 mice were protected from a range of challenge doses (approximately 10–1000 pfu or 300–30,000 LD_{50}) in the absence of adjuvant in a dose-dependent manner [UNPUBLISHED DATA, 84]. Adding QS-21 or RIBI adjuvant to the eVLP vaccine regimen completely protected mice and guinea pigs from challenge, even after a single vaccine dose [UNPUBLISHED DATA, 77]. Ebola VLP vaccination of mice and guinea pigs completely prevented viremia (tested on day 7) and clinical symptoms for at least 28 days following EBOV challenge [77,84,85].

Ebola VLPs are highly immunogenic, and in mice eVLP injection activates B- and T-lymphocytes within a few days [84]. Mice and guinea pigs vaccinated with eVLP produce EBOV-specific antibodies, including neutralizing antibodies, and the total serum antibody levels correlate with the vaccine dosage [77,84–86]. Vaccination with eVLP activates $CD4^+$ and $CD8^+$ T-cells in mice, and generates $CD4^{+-}$ and $CD8^+$ EBOV-specific T-cells [84,85]. Splenocytes from VLP-vaccinated mice secrete interferon (IFN)-γ and not interleukin (IL)-4 in mixed lymphocyte reactions, indicating that T-helper (Th)1-type immune responses are generated in response to VLP vaccination [85]. However, a significant rise in EBOV-specific antibodies and the development of cytotoxic T-lymphocytes recognizing NP, VP24 and 35 at 28 days after EBOV infection is observed in surviving eVLP-vaccinated rodents [77,84,85]. These data indicate that eVLP vaccination does not induce sterilizing immunity in rodents.

To examine the mechanisms of VLP-mediated protection, knockout mice were vaccinated with VLPs and then challenged with EBOV. Severe combined immunodeficient (SCID) mice, which lack all the components required for adaptive immunity, do not develop EBOV-specific antibodies and are not protected from EBOV challenge when vaccinated with eVLPs (TABLE 1). B-cell-deficient JH mice vaccinated with eVLPs were not protected from lethal EBOV challenge; in contrast, genetically matched BALB/c mice were completely protected by eVLP vaccination. Similarly, eVLP-vaccinated T-cell-deficient mice succumbed to lethal EBOV challenge, and β2m-deficient mice, which lack $CD8^+$ T-cells, were completely unprotected. However, eVLP vaccination of $CD4^+$ T-cell-deficient mice partially protected them from EBOV challenge. Protection induced by eVLP vaccination required the presence of IFN-γ, but not perforin. Therefore, the production of both $CD8^+$ T-cell and antibody responses after eVLP vaccination is required to mediate protection against lethal EBOV infection [85].

Marburg virus-like particles

Similar to eVLPs, Marburg (m)VLPs composed of the MARV GP and matrix protein VP40 are produced spontaneously in transfected mammalian cells [21]. Guinea pigs vaccinated with mature (m)VLPs in RIBI adjuvant develop high levels of serum virus-specific and -neutralizing antibodies [86]. Vaccinating guinea pigs with mVLP elicits proliferative responses *in vitro* after exposure to MARV, but not EBOV, and proliferation is mediated by MARV-specific $CD4^+$ T-cells *in vitro* [86]. Upon challenge with MARV, mVLP-vaccinated guinea pigs are completely protected from clinical symptoms, viremia and death [77,86]. Musoke-based mVLPs completely protect guinea pigs against viremia and lethal disease after challenge with guinea pig-adapted MARV-Musoke, -Ravn or -Ci67 [UNPUBLISHED DATA]. These data indicate that Musoke-based VLPs may be effective at inducing broad immunity against multiple MARV strains.

Virus-like particles as a pan–filovirus vaccine

Considering the promiscuity of the filovirus proteins in assembly, as well as the fact that MARV-Musoke-derived VLPs were observed to protect against heterologous MARV challenge, it was important to determine whether the VLPs had the potential to protect against diverse filovirus strains. In guinea pigs, eVLP vaccination induced high EBOV-specific antibody responses, but only a small amount of heterologous antibodies towards MARV [77,84,86]. Furthermore, eVLP vaccination failed to crossprotect against MARV challenge in guinea pigs [77,86]. Similarly, mVLP vaccination of guinea pigs induced only minimal EBOV-specific antibodies and did not protect them against EBOV challenge [77,86]. Hybrid VLPs, containing various combinations of EBOV and MARV proteins, provided an effective tool to examine the roles of GP

Table 1. Immune responses and survival of eVLP-vaccinated knockout mice.

Genotype*	Immune deficiencies	Vaccine‡	Geometric mean titer§	Survivors/total¶	MTD#
C57Bl/6	Wild type	eVLPs	31711	18/20	N/A
		PBS	<33	0/20	7.8 ± 0.69
BALB/c	Wild type	eVLPs	32346	9/10	N/A
		PBS	<33	0/10	6.1± 0.85
Scid (BALB/c)	B- and T-cells	eVLPs	<33	0/10	5.5 ± 0.52
		PBS	<33	0/10	5.6 ± 0.53
Jh (BALB/c)	B-cells	eVLPs	<33	0/10	6.2 ± 0.44
		PBS	<33	0/10	6.1 ± 0.32
βδTCR (C57)	T-cells	eVLPs	460	0/10	7.2 ±0.42
		PBS	<33	0/10	6.9 ± 0.57
CD4 (C57)	CD4 T-cells	eVLPs	5196	5/10	8.4 ± 0.54
		PBS	<33	0/10	7.0 ± 0.47
β2m (C57)	CD8 T-cells	eVLPs	15924	0/10	10.2 ± 1.4
		PBS	<33	0/10	7.1 ± 0.32
IFN-γ (C57)	IFN-γ	eVLPs	11523	2/15	9.6 ± 1.1
		PBS	<33	0/15	7.0 ± 0
Perforin (C57)	Perforin	eVLPs	36955	15/15	N/A
		PBS	<33	0/15	6.3 ± .0.73

*Genotype of mice used are indicated along with the strain background in parentheses.
‡A total of 10 µg of eVLPs or PBS mixed with QS-21 adjuvant were administered on days 0 and 21.
§Geometric mean titer of EBOV-specific antibodies as measured by ELISA.
¶After challenge with 1000 pfu of mouse-adapted EBOV 6 weeks following the last vaccination.
#MTD following EBOV challenge in days ± standard deviation.
EBOV: Ebola virus; eVLP: Ebola VLP; IFN: Interferon; MTD: Mean time to death; PBS: Phosphate-buffered saline; Pfu: Plaque-forming unit; N/A: Not applicable.

and VP40 in protective immunity against filoviruses. Guinea pigs vaccinated with VLPs containing the homologous GP were protected (≥ 90%) from filovirus challenge [77]. For example, a single injection of eVLP or hybrid VLPs containing EBOV GP and MARV VP40 administered concomitantly with RIBI adjuvant significantly protected test subjects from EBOV challenge, but not MARV challenge. Similarly, mVLP or hybrid VLPs containing MARV GP and EBOV VP40 completely protected MARV-challenged, but not EBOV-challenged guinea pigs [77]. Together, these data indicate that GP is the critical protective antigen in the VLPs, and that VP40 is likely required only for maintaining the filamentous VLP structures. In addition, VP40-only VLPs did not protect vaccinated mice from a lethal EBOV challenge, which further substantiates these data [UNPUBLISHED DATA].

Hybrid VLPs did not provide broad protection against both EBOV and MARV; however, coadministration of a mixture of eVLP and mVLP did protect guinea pigs against EBOV and MARV challenge [77]. Before challenge, the guinea pigs vaccinated with both eVLP and mVLP developed high antibody titers against both EBOV and MARV, and the titers generated to the homologous antigen were similar to those in animals vaccinated with eVLP or mVLP alone [77]. Therefore, vaccinating animals simultaneously with both antigens did not interfere with their ability to initiate humoral responses to the individual antigens [77]. High levels of protection are observed after vaccination with the mixture of eVLP and mVLP, similar to the protection observed in the groups of animals vaccinated with eVLP or mVLP alone and challenged with the homologous virus [77]. This work is the first demonstration of an effective pan-filovirus vaccine, and justifies the further testing of VLPs as a pan-filovirus vaccine in nonhuman primates. In addition, future work will focus on testing the efficacy of VLPs that incorporate multiple GP molecules onto VP40 from a single species.

Innate immune responses to virus-like particles

The key for developing effective adaptive immunity to pathogens is the rapid detection of the microbe and subsequent activation of the host innate immune response. Monocytes,

macrophages and DCs, also known as antigen-presenting cells, are central in both the activation of innate immunity and initiation of adaptive immunity. Antigen-presenting cells drive immune responses by inducing cytokines and chemokines, antigen presentation, interactions with B-, T- and NK cells, and direct cytotoxic activity against target cells [87,88]. EBOV and MARV readily infect monocytes, macrophages and DCs and replicate rapidly within these cells to produce large amounts of progeny virus (FIGURE 3A) [32–34,36,37,89,90]. Filovirus-infected DCs fail to undergo maturation or activation and do not initiate appropriate NK, B- and T-cell responses following EBOV or MARV infection [34,89]. The demise of the innate immune system and the failure to quickly activate adaptive immune responses likely result in uncontrolled disseminated filovirus infection of the host [34,37,89]. The activation of filovirus-infected macrophages may also be impaired, although the response of monocytes and macrophages to filovirus infection is controversial [32–34,36].

In contrast to live EBOV and MARV, Ebola and Marburg VLPs are highly immunogenic in rodents and primate species (FIGURE 3B), potently activating *in vitro* murine bone marrow-derived DCs, human monocyte-derived DCs and human monocytes [84,91,92]. In order to determine whether initiation of these rapid innate immune responses were sufficient for protection, mice were administered eVLPs shortly before injection with a lethal dose of EBOV. Mice injected intraperitoneally or intramuscularly with a single dose of eVLP 1–3 days before challenge demonstrated 80–100% protection, indicating that eVLPs induced potent innate immune responses [42].

The rapid immunity caused by eVLP injection required NK cells, as demonstrated by antibody depletion of NK cells of normal mice and NK cell-knockout mice [42]. Since NK cells do not become activated and are specifically depleted from the blood of EBOV infected-monkeys and mice, this study further explored the role of NK cells in EBOV infections [UNPUBLISHED DATA,42,93]. In contrast to live EBOV, NK cells treated with VLPs exhibit enhanced cytokine secretion and cytolytic activity [42]. Furthermore, adoptive transfer of NK cells treated with eVLPs protects naive mice against EBOV infection and the mechanism of this protective innate immunity requires perforin, but not IFN-γ [42]. Interestingly, VP40 VLPs are sufficient to induce NK cell responses and to provide protection from infection in the absence of the viral GP [42]. The specific receptor(s) and signals required by NK cells to respond to VLPs are currently being investigated by the authors of this review.

The immunogenicity of Ebola and Marburg VLPs in human and murine DCs and NK cells already described points toward their role in understanding filovirus interactions with multiple components of the host immune response. Understanding the differences between the responses to VLPs and live filoviruses could lead to the targeted development of strategies to control filovirus infections. Therefore, this work may ultimately lead to identifying novel and effective immunotherapeutics against these and other devastating and highly pathogenic viruses.

Dissecting the filovirus life cycle using virus-like particles

There are certain advantages to using VLPs for viral life cycle studies compared with live filoviruses. Studies involving VLPs can be conducted in Biosafety Level (BSL)-2 conditions as opposed to working with live EBOV or MARV in BSL-4 laboratories, which significantly decreases the practical difficulties and allows for a broader variety of techniques to be applied when investigating questions regarding the filovirus life cycle. Current safety protocols require EBOV- and MARV-infected cells to be treated with harsh fixation methods, and irradiation before immunomicroscopy may be performed. These treatments result in less than optimal cellular morphology, making specific cellular compartments difficult to distinguish. In contrast, mild detergents for fixation or live studies can be performed when examining cells either producing or exposed to VLPs.

Current understanding of filoviral entry will probably be improved using VLPs, as they closely resemble filoviruses and could identify the receptor for viral entry. Pseudotyped vesicular stomatitis or HIV viruses incorporating GP into their envelope have been used to study the binding and entry mechanisms of EBOV and MARV [76,94]. However, it has been suggested that filoviral entry and budding is context-dependent and may require cofactors present in the filoviral envelope, as well as the correct confirmation of GP on the virion surface [75,95]. The envelope and virion structure of pseudotyped viruses is probably different to that of filoviruses, whereas VLPs appear to maintain the morphology of authentic filovirus particles. VLPs can be generated with multiple and different combinations of viral proteins, and are a useful tool for determining which viral or host cell proteins enhance fusion and entry into the cell. Additionally, as VLPs do not carry any viral RNA into the cell, studies using VLPs may provide valuable insights into early events required for initial infection. The use of VLPs likely better represents the initial infectious process because the effects of virion-associated proteins and nascent protein expression are eliminated.

Development of therapeutics and vaccines can be facilitated by identifying key host cell proteins involved in replication and the viral life cycle. For example, proteomics could be used to identify host cell proteins involved in the entry, assembly, and release of the virion. It has been demonstrated that EBOV and MARV require lipid rafts for the efficient assembly of virus particles [20]. A comparison of the lipid rafts of cells transfected with select viral proteins and VLPs could, therefore, be used to determine the host cell and viral membrane composition following expression of viral proteins during filovirus assembly budding. Furthermore, determining the signaling events within the membranes by studying protein modifications within the lipid rafts, such as acylation and phosphorylation, would identify further host cell proteins critical for supporting viral replication. These studies might help determine binding and entry mechanisms, replication requirements, as well as identify the viral (co)-receptor(s).

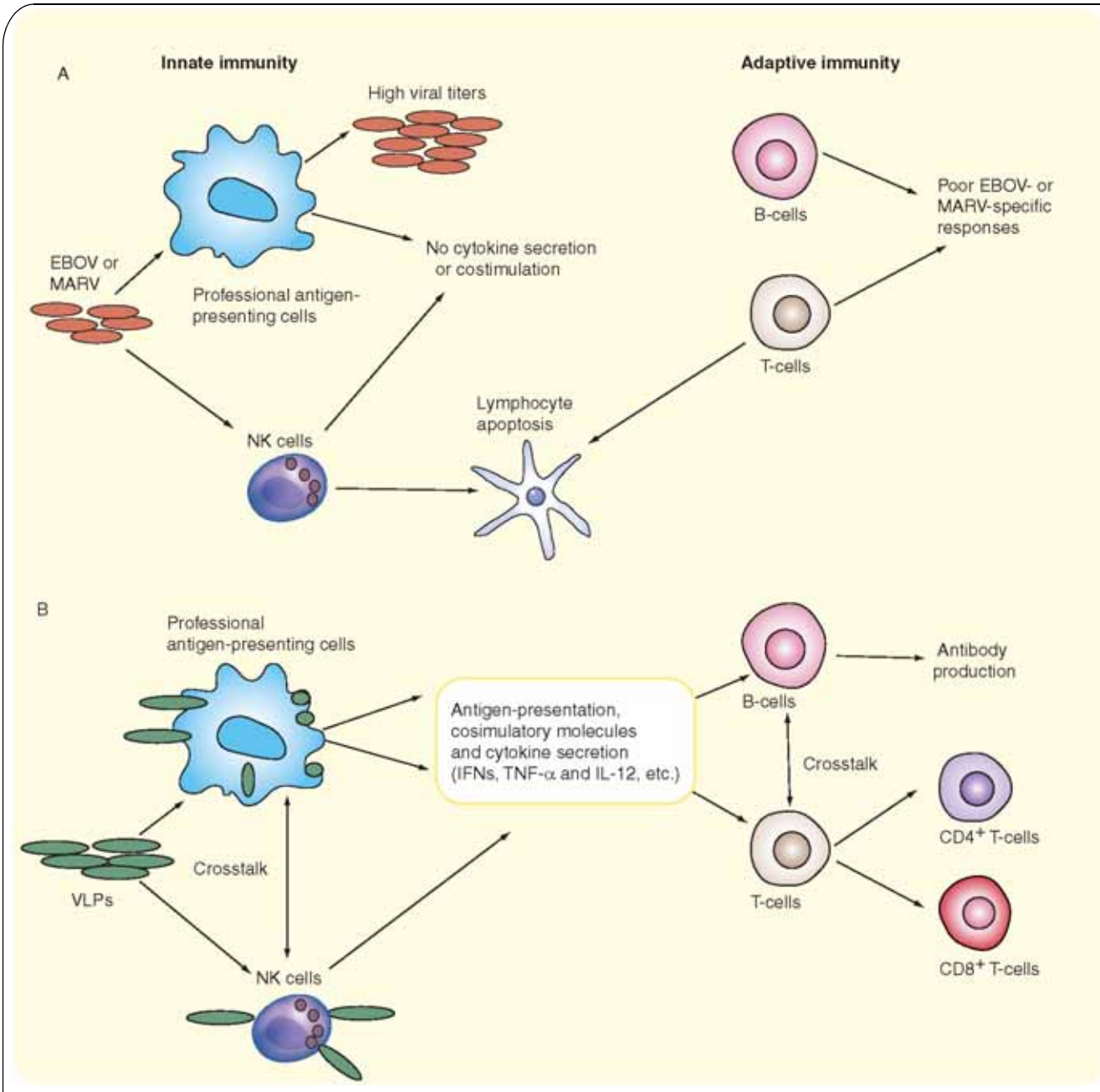

Figure 3. Model of innate and adaptive immune responses following exposure to filoviruses or filovirus-like particles. (A) Poor immune responses occur in individuals that succumb to filovirus infections. EBOV or MARV infection of monocytes, macrophages and dendritic cells (also known as professional antigen-presenting cells) results in the production of large amounts of virus. Critical members of the innate immune responses such as macrophages, dendritic cells, and NK cells fail to appropriately respond to filovirus infections. Without direction from the innate immune response, B- and T-cells do not become fully alerted and EBOV- and MARV-specific adaptive responses lag behind the rapid viral spread. Furthermore, widespread lymphocyte apoptosis (specifically NK and T-cells) occurs within lymphoid tissues. **(B)** Ebola and Marburg VLPs induce strong innate and adaptive immune responses. Exposure to VLPs induces maturation and activation of macrophages, dendritic cells, and NK cells. Induction of antigen presentation, expression of costimulatory molecules, and cytokine and chemokine secretion directs the subsequent B and T-cell responses, resulting in the production of Ebola and Marburg virus-specific antibodies, $CD4^+$ and $CD8^+$ T-cells. Additional crosstalk occurs via cytokine secretion and physical contact between multiple cell types including NK and dendritic cells, as well as B- and T-cells, to further direct and control the immune response.
EBOV: Ebola virus; IFN: Interferon; IL: Interleukin; MARV: Marburg virus; NK: Natural killer; TNF: Tumor necrosis factor; VLPs: Virus-like particles.

At present, Ebola and Marburg VLPs are used most frequently for studying the mechanisms of virus assembly. Recent studies using VLPs have revealed contributions of both viral proteins and cellular components that are required for the viral assembly process. These studies have been reviewed thoroughly, and an in-depth discussion is

beyond the scope of this review [22,75]. Studies to understand the life-cycle mechanisms will provide critical information about the host and viral proteins important for these processes, thereby providing promising targets for novel viral therapies and vaccines.

Filovirus-like particles as a diagnostic tool

Several accurate diagnostic tests are available for EBOV and MARV including virus isolation, immunoglobulin (Ig)G and IgM antibody tests, immunofluorescent antibody tests, antigen-capture enzyme-linked immunosorbent assay (ELISA) along with western blot confirmation, radioimmunoprecipitation assays (RIPAs), reverse transcriptase PCR, and fluorogenic 5´-nuclease assay [4]. Although virus isolation is considered to be a necessary stage in the confirmation of a filovirus outbreak, isolation of filoviruses can take up to 2 weeks, and requires a BSL-4 facility; furthermore, the sample must be correctly preserved using a cold chain. An antigen-capture ELISA together with western blot is quick, easy and robust, and adaptable to large numbers of samples. VLPs can be quickly, safely and easily produced as antigens for use in ELISA and western blotting assay for the detection of EBOV and MARV infections in the field.

Summary & conclusions

Members of the family *Filoviridae* cause severe and rapidly progressive hemorrhagic fever, resulting in high mortality rates. Unfortunately, there are currently no vaccines or treatment options available for filovirus infections. A number of experimental vaccination strategies are being explored, including soluble viral proteins, DNA vaccines, replicons and a combination of DNA and replication-deficient adenovirus preparations. Given the importance of both the cellular and humoral immune response for protection against filoviruses, VLPs represent an excellent vaccine candidate [60,64,70,96]. Studies in rodents have demonstrated that vaccination with EBOV or MARV VLPs provides almost uniform protection from lethal homologous challenge. Although neither homologous nor hybrid VLPs crossprotect against heterologous challenge, simultaneous vaccination with both EBOV and MARV VLPs can protect against either EBOV or MARV infection. These candidate vaccines are currently being tested for immunogenicity and efficacy in nonhuman primates. Preliminary data indicate that both eVLPs and mVLPs are highly immunogenic in monkeys and stimulate virus-specific humoral and cellular responses [UNPUBLISHED OBSERVATIONS]. Besides their use as vaccines, VLPs are being used to better understand the immune responses to filoviruses, with the aim of developing immunotherapeutics for treating EBOV and MARV infections. In addition, the EBOV and MARV VLPs are being used to investigate the life cycle of these viruses with the ultimate goal of developing rationally designed therapeutics. Thus, in addition to their use as a vaccine, VLPs are a tool for the dissection of filovirus pathogenesis, immunology, replication and assembly requirements.

Expert commentary

A variety of vaccine approaches have been used against lethal filovirus infections. The most successful vaccines so far are based on viral vectors, including adenovirus, VEE and vesicular stomatitis virus. It appears that proper presentation of viral proteins, as well as vaccine dose, are critical qualities for successful filovirus vaccines. Induction of both antibodies, to stave off the early virus infection, and cytotoxic T-cells, to destroy virus-infected cells, are required for immunity against filoviruses. The VLPs are a promising candidate vaccine, probably due to the proper presentation of viral proteins to the immune system. The filovirus-like particles possess several advantages over other candidate vaccines, including presentation of antigen in its native form, lack of interference by a vector backbone, absence of prior immunity to the vector and, potentially the absence for the need of adjuvant. For manufacturing purposes, VLPs are safe and easy to produce in large quantities. The VLPs have proven to be successful vaccines for filovirus infection in rodent models, but their efficacy in nonhuman primates has yet to be demonstrated. It is possible that incorporation of additional filovirus proteins into VLPs may further improve the immunogenicity of the particles, especially across genetically diverse primates and humans. Vaccination with combinations of filovirus GP and NP have protected nonhuman primates against EBOV and MARV infections, and NP is known to induce strong cytotoxic T-cell responses [64–66,68,70,96,97]; therefore, adding NP to a VLP-based vaccine may further expand the CTL repertoire and stimulate more robust CTL responses. The role of the other viral proteins, including VP24, 30 and 35, is unknown, and although they are immunogenic, their protective effects are less robust [64,65,96]. Based on their safety profile, immunogenicity, and success to date, the VLPs are a strong candidate for use as a filovirus vaccine in humans.

Five-year view

The results obtained thus far for EBOV and MARV VLPs provide the groundwork for future studies to evaluate the efficacy of VLPs for protection against both MARV and EBOV in nonhuman primates. Work to identify mechanisms of pathogenesis and immunity in mice is ongoing. These studies should help identify the protective T-cell epitopes against filovirus proteins and identify possible surrogates of immunity for VLP vaccines. Within the next few years, studies with eVLPs and mVLPs will be performed against all the diverse strains of EBOV and MARV to evaluate their vaccine potential, determine the best vaccination route, establish correlates of immunity, and understand the role of different arms of the immune system in protective immunity against these viruses. Work performed in rodents will need to be expanded to nonhuman primates, and ultimately to humans, and these studies are already beginning to take place. Efficacy testing of VLPs delivered intranasally and orally are also a priority, and mucosal administration would provide an option for needleless delivery, especially for use in

Africa. Tests of monovalent and multivalent VLP vaccines for filoviruses will be performed in nonhuman primates within the next 5 years. A clear profile regarding the minimal level of protective T-cell and antibody responses, as well as any other correlates of protection, will have to be developed in order to determine the level and duration of protection conferred by the vaccine before FDA approval will be obtained. The developments will pave the way for producing a safe, multiagent filovirus-like particle-based vaccine for use in humans.

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Key issues

- Filovirus virus-like particles containing several filovirus proteins can be produced easily and efficiently in mammalian cells.
- The filovirus virus-like particles effectively interact with cells of the innate immune system, including dendritic cells, natural killer cells and macrophages, and may be used to understand how authentic filoviruses escape detection.
- Virus-like particles induce virus-specific humoral and cell-mediated immunity following virus-like particle vaccination and protect mice and guinea pigs against lethal filovirus challenge.
- The efficacy of a filovirus vaccine cannot be ethically evaluated in humans; thus, studies to determine the protective capabilities of virus-like particle vaccines will have to be performed in surrogate models, such as rodents and nonhuman primates.
- Virus-like particle vaccines have been well-tolerated and are effective in healthy, human volunteers. The safety profile of virus-like particles may also permit their use in immunocompromised individuals.
- Virus-like particles have uses other than as vaccines, and are currently being used to learn about the virus life cycle, as well as being developed as a diagnostic tool for filovirus infections.

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Affiliations

- *Kelly L Warfield*
  United States Army Medical Research Institute of Infectious Diseases,
  Fort Detrick, MD 21702–5011, USA
  Tel.: +1 301 619 3414
  Fax: +1 301 619 2348
  kelly.warfield@det.amedd.army.mil
- *Dana L Swenson*
  United States Army Medical Research Institute of Infectious Diseases,
  Fort Detrick, MD 21702–5011, USA
  Tel.: +1 301 619 5112
  Fax: +1 301 619 2348
  dana.swenson@det.amedd.army.mil
- *Gretchen Demmin*
  United States Army Medical Service Corps,United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702–5011, USA
  Tel.: +1 301 619 6731
  Fax: +1 301 619 2290
  gretchen.demmin@det.amedd.army.mil
- *Sina Bavari*
  Target Identification and Translational Research United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702–5011, USA
  Tel.: +1 301 619 3414
  Fax: +1 301 619 2348
  sina.bavari@det.amedd.army.mil or bavaris@ncifcrf.gov